

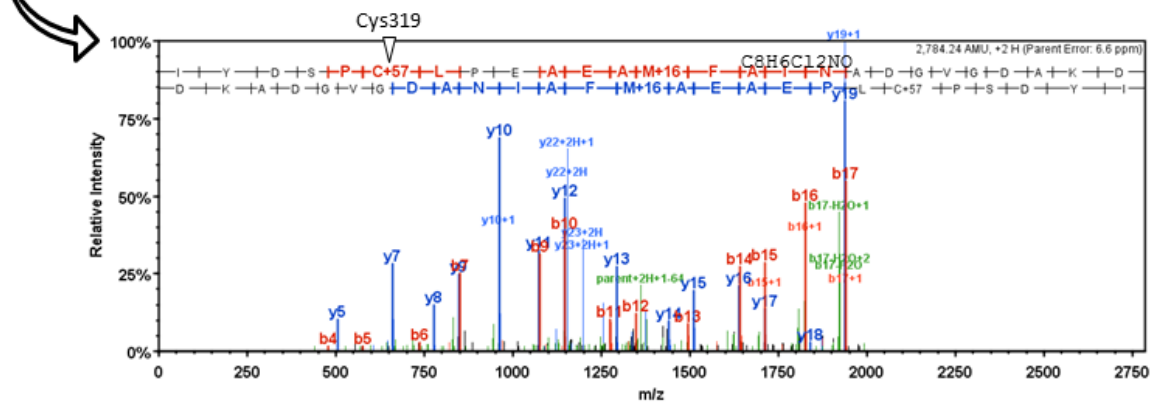
Supplemental Figure 1a:

No RI-1 (79% of HsRAD51 sequence covered):

Sequence coverage  
Carbamidomethyl  
Oxidation  
Acetyl

1	MAMQMQL	LEAN	ADTSVEEESF	GPQPISR	LEQ	CGINANDVKK	LEEAGFHTVE	AVAYAPKKEL
61	INIKGISEAK	ADKILAEAAK	LVP	MGFTTAT	EFHQRRSEII	QITTGSKELD	KLLQGGIETG	
121	SITEMFGEFR	TGKTQICHTL	AVT	CQLPIDR	GGGEGKAMYI	DTEGTFRPER	LLAVAERYGL	
181	SGSDVLDNVA	YARAFNTDHQ	TQLLYQASAM	MVESRYALLI	VDSATALYRT	DYSGRGELSA		
241	RQMHLARFLR	MLLRLADEFG	VAVVITNQVV	AQVDGAAMFA	ADPKPIGGN	IIAHASTTRL		
301	YLRKGRGETR	ICKIYDSPCL	PEAEAMFAIN	ADGVGDAKD				

IYDSPCLPEAEAMFAINADGVGDAK	(95%)
IYDSPCLPEAEAMFAINADGVGDAK	(95%), carbamidomethyl (+57) x129
IYDSPCLPEAEAMFAINADGVGDAKD	(95%), carbamidomethyl (+57) x318
ICKIYDSPCLPEAEAMFAINADGVGDAK	(95%) x3
ICKIYDSPCLPEAEAMFAINADGVGDAKD	(95%)



## Supplemental Figure 1b:

10  $\mu$ M RI-1 (81% of HsRAD51 sequence covered):

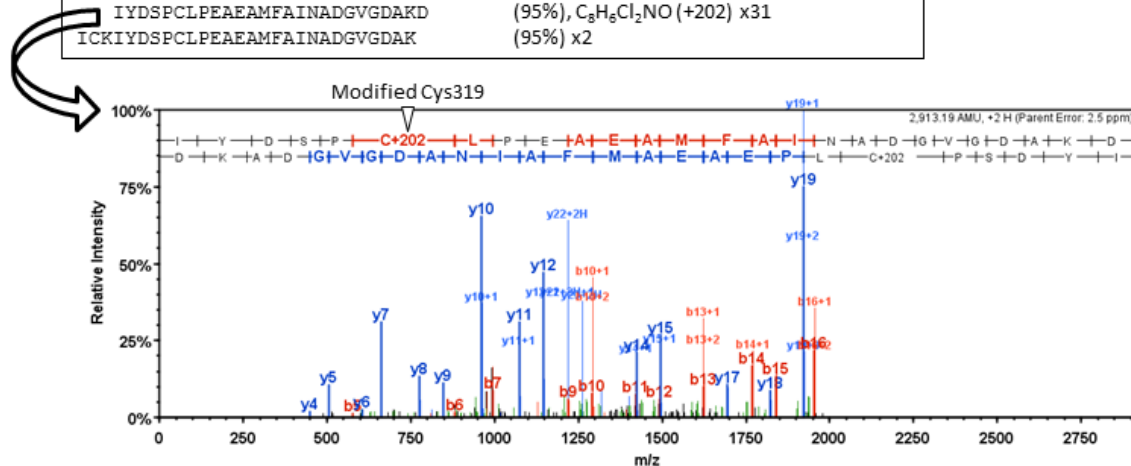
Sequence coverage  
Carbamidomethyl  
Oxidation  
Acetyl  
 $C_8H_6Cl_2NO$  or carbamidomethyl

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1  MAMQMQLLEAN  ADTSVEEESF  GPQPISRLEQ  CGINANDVKK  LEEAGFHTVE  AVAYAPKKEL
61  INIKGISEAK  ADKILAEAAK  LVPMGFTTAT  EFHQRRSEII  QITTGSKELD  KLLQGGIETG
121  SITEMFGEFR  TGKTQICHTL  AVTCQLPIDR  GGGEGKAMYI  DTEGTFRPER  LLAVAERYGL
181  SGSDVLDNVA  YARAFNTDHQ  TQLLYQASAM  MVESRYALLI  VDSATALYRT  DYSGRGELSA
241  RQMHLARFLR  MLLRLADEFG  VAVVITNQVV  AQVDGAAMFA  ADPKRPIGGN  IIAHASTRRL
301  YLRKGRGETR  ICKIYDSPCL  PEAEAMFAIN  ADGVGDAKD

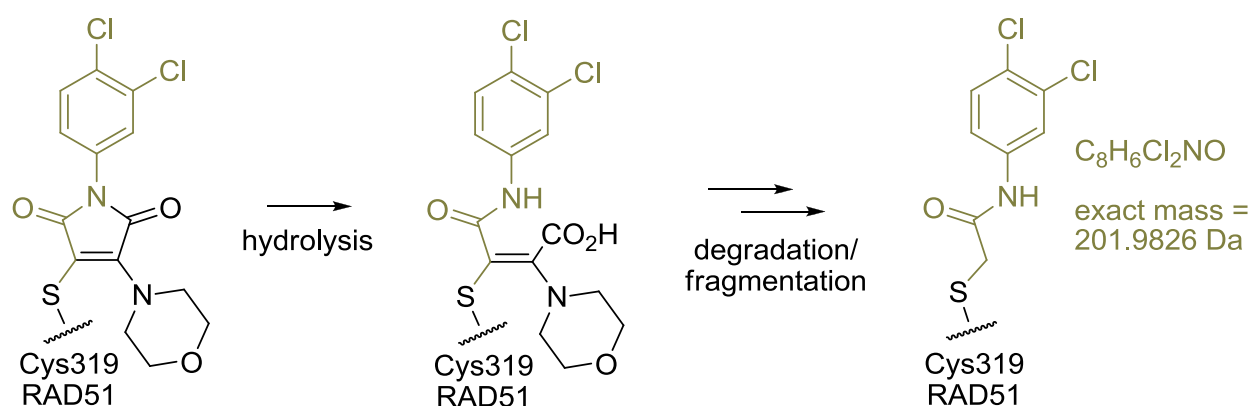
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IYDSPCLPEAEAMFAINADGVGDAK (95%), carbamidomethyl (+57) x191  
 IYDSPCLPEAEAMFAINADGVGDAKD (95%), carbamidomethyl (+57) x207  
 IYDSPCLPEAEAMFAINADGVGDAKD (95%),  $C_8H_6Cl_2NO$  (+202) x31  
 ICKIYDSPCLPEAEAMFAINADGVGDAK (95%) x2



**Supplemental Figure 1: Mass spectrometry demonstrates that RI-1 forms a covalent adduct at C319 of HsRAD51 protein.** The HsRAD51 protein was incubated with 10  $\mu$ M RI-1 (B) or buffer alone (A) for 30 minutes at 37° C, isolated on a SDS PAGE, digested with trypsin, and analyzed via LC-MS/MS. Peptide sequences identified are highlighted yellow within the RAD51 protein sequence, and amino acid modifications are highlighted with other colors as indicated. The peptide sequences identified corresponding to RI-1 adducts are listed within the below box; for each the probability that the peptide has been properly identified is shown in parentheses, followed the composition of any observed chemical modification (+MW in parentheses), followed by the number of times that peptide was observed. MS spectral peaks are displayed for the peptide of greatest interest, which contains the RI-1 adduct.

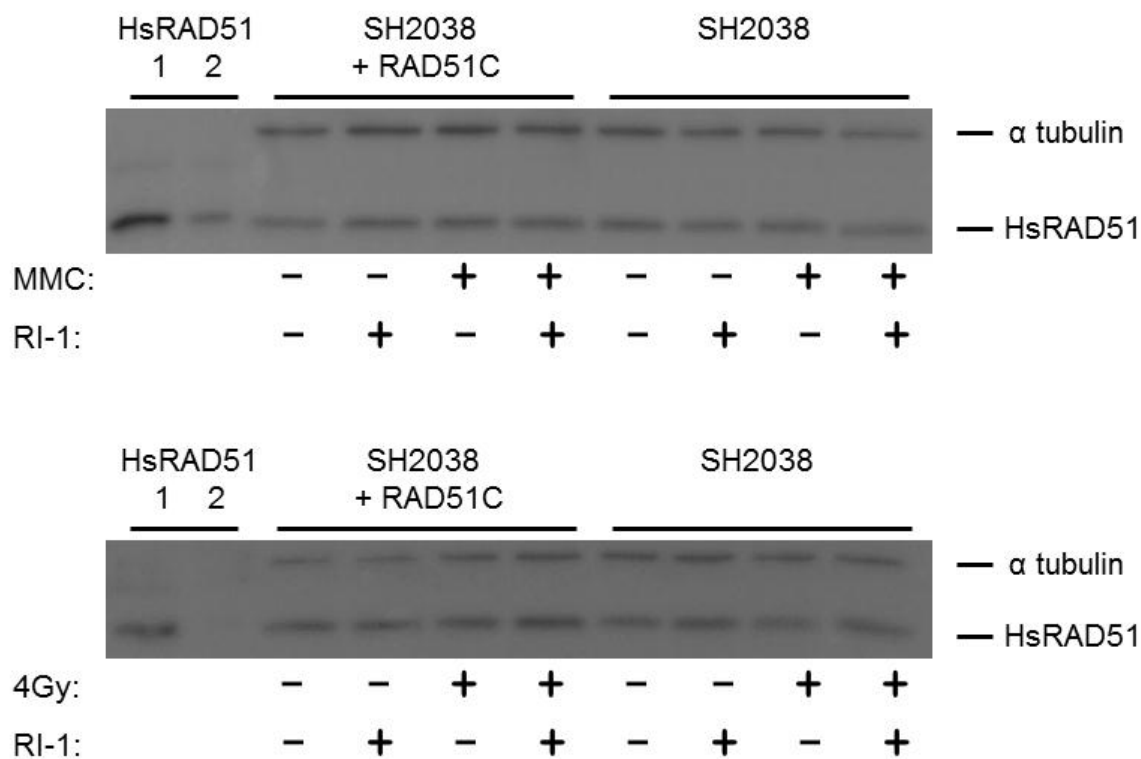
**Supplemental Figure 2:**



**Supplemental Figure 2: Proposed explanation for the 202 Dalton adduct on HsRAD51**

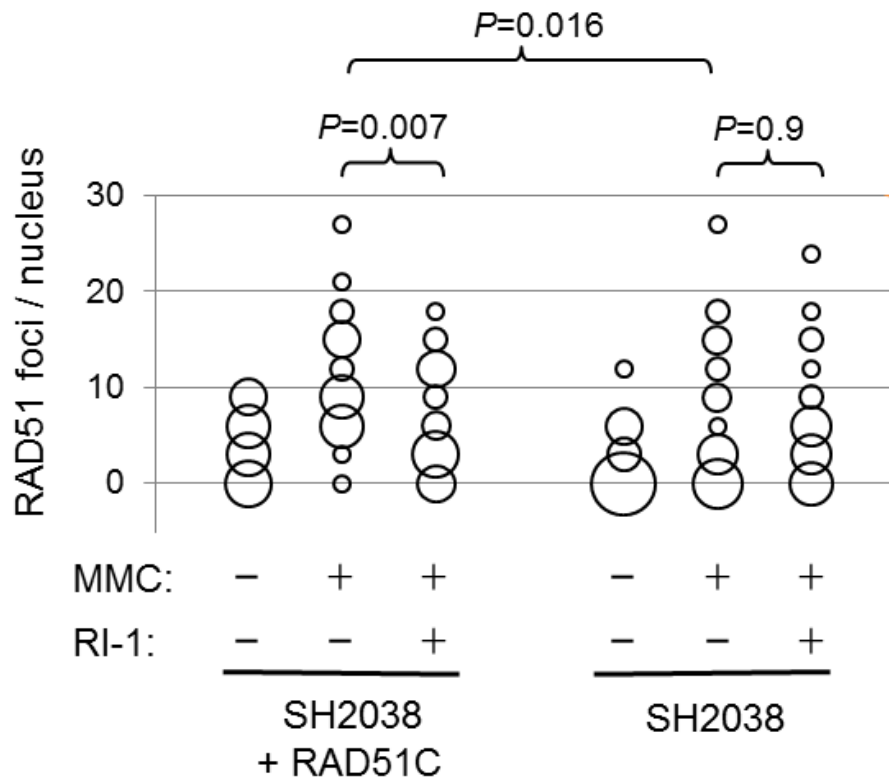
**protein.** Halomaleimides have been shown to react with cysteine residues via a Michael reaction, and the pH of the reaction has been demonstrated to mediate subsequent hydrolysis of the imide. Additional decomposition and/or fragmentation may lead to the adduct proposed above, which would account for the observed difference in molecular weight of the peptide fragment containing C319.

**Supplemental Figure 3:**



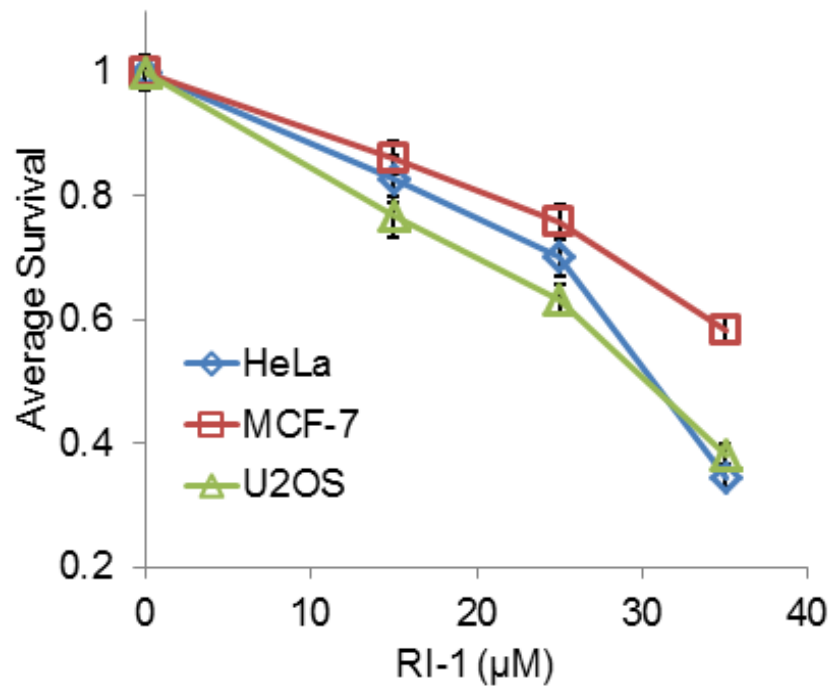
**Supplemental Figure 3: RI-1 does not impact RAD51 protein levels.** Western blots were performed on whole cell extracts (7.5 µg protein per lane), which were prepared from cells treated identically to those described in Figure 5. Purified HsRAD51 positive control consisted of 9.75 ng in lane 1 and 3.25 ng in lane 2.

**Supplemental Figure 4:**



**Supplemental Figure 4: RI-1 disrupts the formation of RAD51 foci after MMC in S/G2 cells.** Immortalized human fibroblasts (SH2038 +/- RAD51C) were incubated for 8 hours in media containing 150 nM mitomycin C (MMC) and/or 30  $\mu$ M RI-1, and cells were allowed to incorporate the thymidine analog EdU for the last three hours of this incubation. Cells subsequently harvested and stained for RAD51 and Edu as described in the methods. Twenty seven randomly selected EdU-positive nuclei per treatment group were examined and quantified. The size of each 'bubble' represents the number of cells having a given number of foci/nucleus.

**Supplemental Figure 5:**



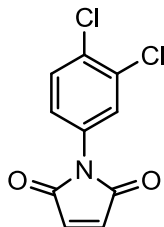
**Supplemental Figure 5: RI-1 exerts single-agent toxicity in human cancer cell lines.** Three human cancer cell lines were incubated for 24 hours in media containing varying concentrations RI-1. Cells were then allowed to grow in drug-free media for an additional 4-6 days. Average survival is shown for each condition.

## **Supplemental Methods:**

### **General Information for RI-1 Synthesis:**

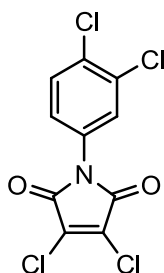
**General Information for Synthetic Procedures:**  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were obtained using a Bruker spectrometer with TMS as an internal standard. The following standard abbreviations indicating multiplicity were used: s = singlet, d = doublet, t = triplet, m = multiplet, and br = broad. HRMS experiments were carried out using a Shimadzu IT-TOF instrument with MeCN and  $\text{H}_2\text{O}$  spiked with 0.1% formic acid as the mobile phase. Reaction progress was monitored by TLC using precoated silica gel plates (Merck silica gel 60 F254, 250  $\mu\text{m}$  thickness). Automated column chromatography was performed using the CombiFlash Rf apparatus available from Teledyne ISCO and prepacked cartridges (50 g) loaded with Merck silica gel (40–60 mesh) along with the following conditions: Gradient: 100% hexane, 5 min; 0–50% EtOAc/hexane, 25 min; 50% EtOAc/hexane, 5 min. Flow rate = 40 mL/min with wavelength monitoring at 254 and 280 nm. Preparatory HPLC was carried out using a Shimadzu preparative liquid chromatograph with the following specifications: Column: ACE 5 AQ (150  $\times$  21.2 mm) with 5  $\mu\text{m}$  particle size. Gradient: 25–100% MeOH/ $\text{H}_2\text{O}$ , 30 min; 100% MeOH, 5 min; 100–25% MeOH/ $\text{H}_2\text{O}$ , 4 min; 25% MeOH/ $\text{H}_2\text{O}$ , 1 min. Flow rate = 17 mL/min with wavelength monitoring at 254 and 280 nm. Both solvents were spiked with 0.05% TFA. Resin bound bicarbonate was used to neutralize residual trifluoroacetic acid remaining from preparatory HPLC purification. Analytical HPLC was carried out using an Agilent 1100 series instrument with the following specifications: Column: Luna 5  $\mu$  C18(2) 100A (150  $\times$  4.60 mm) with 5  $\mu\text{m}$  particle size. Flow rate = 1.4 mL/min with wavelength monitoring at 254 nm. Gradient: 10–100% MeOH/ $\text{H}_2\text{O}$ , 18 min; 100% MeOH, 3 min; 100–10% MeOH/ $\text{H}_2\text{O}$ , 3 min; 10% MeOH/ $\text{H}_2\text{O}$ , 5 min. Both solvents were spiked with 0.05% TFA.

**1-(3,4-Dichlorophenyl)-1*H*-pyrrole-2,5-dione (1):**



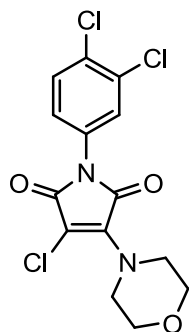
Furan-2,5-dione (2.50 g, 25.5 mmol) was placed in a two-neck round bottomed flask fitted with a condenser and dissolved in anhydrous Et<sub>2</sub>O (50 mL) under argon atmosphere. Upon complete dissolution, 3,4-dichloroaniline (4.13 g, 25.5 mmol) was dissolved in anhydrous Et<sub>2</sub>O (10 mL) and added dropwise at RT resulting in the formation of a white precipitate. Stirring was continued for 1.5 h at RT after which the reaction was concentrated *in vacuo*, cooled in an ice bath and filtered. The filter cake was then added to a solution of NaOAc (2.08 g, 25.5 mmol) in acetic anhydride (20 mL) at RT after which the reaction was heated to 100 °C and stirred for 30 min. The reaction was allowed to cool to RT and then poured into ice water (50 mL). The organic products were extracted with EtOAc (3 x 20 mL), washed with H<sub>2</sub>O (3 x 20 mL), brine (15 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated *in vacuo*. The title compound was purified using automated column chromatography and isolated as a light-yellow solid (5.63 g, 91%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.55 (m, 2H), 7.24 (d, *J* = 9.1 Hz, 1H), 6.89 (s, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 168.3, 134.0, 132.7, 131.5, 130.3, 130.2, 127.1, 124.5. ESI-HRMS: calc. for C<sub>10</sub>H<sub>5</sub>Cl<sub>2</sub>NO<sub>2</sub>: [M+H]<sup>+</sup> = 241.9770 *m/z*, found: [M+H]<sup>+</sup> = 241.9762 *m/z*.

**3,4-Dichloro-1-(3,4-dichlorophenyl)-1*H*-pyrrole-2,5-dione (2):**



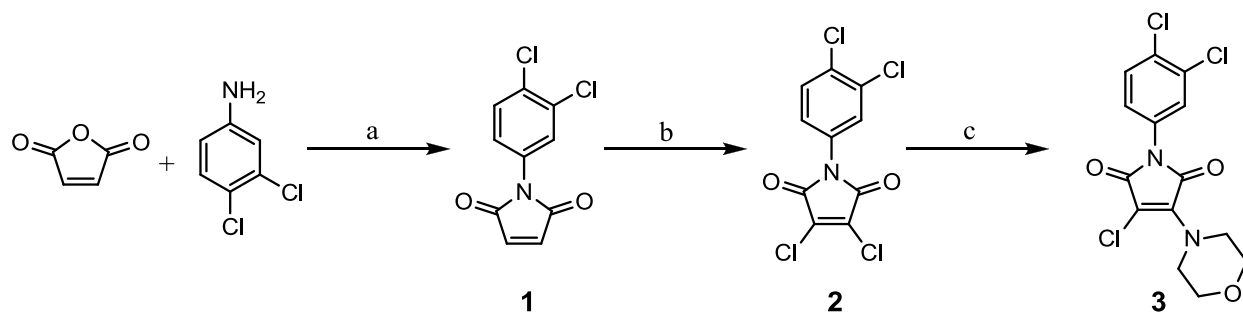
1-(3,4-Dichlorophenyl)-1*H*-pyrrole-2,5-dione **1** (2.00 g, 8.26 mmol) was placed in a round bottomed flask and dissolved in thionyl chloride (5.00 mL) under argon atmosphere. The reaction was cooled to 0 °C in an ice bath and then pyridine (1.40 mL, 17.4 mmol) was added dropwise. The reaction was stirred for 15 min at 0 °C after which it was removed from the ice bath and heated to reflux for 1 h. The reaction was then cooled to RT and the excess thionyl chloride was removed *in vacuo*. The resulting residue was dissolved in CHCl<sub>3</sub> (40 mL), washed with 1N HCl (3 x 10 mL), brine (15 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated *in vacuo*. The title compound was purified by recrystallization from CHCl<sub>3</sub> and isolated as a beige solid (1.16 g, 45%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.57 (m, 2H), 7.29 (m, 1H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 161.4, 133.9, 133.4, 132.9, 131.0, 129.8, 127.5, 124.8. ESI-HRMS: calc. for C<sub>10</sub>H<sub>3</sub>Cl<sub>4</sub>NO<sub>2</sub>: [M+H]<sup>+</sup> = 309.8991 *m/z*, found: [M+H]<sup>+</sup> = 309.8977 *m/z*.

**3-Chloro-1-(3,4-dichlorophenyl)-4-morpholino-1*H*-pyrrole-2,5-dione (3):**



3,4-Dichloro-1-(3,4-dichlorophenyl)-1*H*-pyrrole-2,5-dione **2** (1.00 g, 3.22 mmol) was placed in a round bottomed flask and dissolved in anhydrous DCM at RT under argon atmosphere. Then, morpholine (0.58 mL, 11.6 mmol) was added slowly and the reaction was stirred at RT for 1.5 h resulting in the formation of a yellow precipitate. After the reaction was complete, H<sub>2</sub>O (20 mL), was added and the organic products were extracted with DCM (3 x 20 mL). The combined organic extracts were washed with brine, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated *in vacuo*.

The title compound was purified by preparatory HPLC and isolated as a yellow solid (879 mg, 76%).  $^1\text{H-NMR}$  (400 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  7.76 (d,  $J = 8.5$  Hz, 1H), 7.67 (s, 1H), 7.38 (d,  $J = 7.7$  Hz, 1H), 3.93 (br, 4H), 3.75 (br, 4H).  $^{13}\text{C-NMR}$  (100 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  164.8, 163.9, 142.3, 131.8, 131.4, 131.2, 130.6, 128.8, 127.3, 93.8, 66.8, 48.7. ESI-HRMS: calc. for  $\text{C}_{14}\text{H}_{11}\text{Cl}_3\text{N}_2\text{O}_3$ :  $[\text{M}+\text{H}]^+ = 360.9908$   $m/z$ , found:  $[\text{M}+\text{H}]^+ = 360.9907$   $m/z$ .



Reagents and conditions: (a) i)  $\text{Et}_2\text{O}$ , RT, 1.5 h; ii)  $\text{NaOAc}$ , acetic anhydride, RT  $\rightarrow$  100  $^\circ\text{C}$ , 30 min; b)  $\text{SOCl}_2$ , pyridine, 0  $^\circ\text{C}$   $\rightarrow$  reflux, 1.25 h; c) morpholine,  $\text{DCM}$ , RT, 1.5 h.